Male reproductive health has deteriorated in many countries in recent decades. Male infertility has become a concern since nearly 50% of healthy men are infertile/subfertile (McLachlan and de Kretser, 2001). Infections and inflammation of the male reproductive tract are widely accepted as important etiological factors of subfertility or infertility. Inflammation associated with infections account for almost 15% of the cases of male infertility seen in fertility clinics (Hales et al., 1999; Hong et al., 2004; Schuppe et al., 2008).

Androgens produced in testicular Leydig cells are essential for male sexual differentiation, maintenance of spermatogenesis and secondary sex characteristics (Song et al., 2012). Testicular steroidogenesis takes place almost exclusively in Leydig cells and there is a mounting body of evidence that demonstrate LPS increases proinflammatory cytokines such as TNF-α, IL-1, and IL-6 thereby inhibit testicular Leydig cell steroidogenesis at the level of gene expression of different steroidogenic enzymes (Mealy et al., 1990; Lobo et al., 2010). For example, TNF-α activated nuclear factor-κB (NF-κB) inhibits the transactivation of orphan nuclear receptors, Nur77 and SF-1, thereby resulting in the downregulation of steroidogenic-enzyme gene expression in Leydig cells (McLachlan and de Kretser, 2001; Allen et al., 2004; Song et al., 2012). Despite these startling threats, understanding the biological mechanisms underlying this complex process of infection mediated inhibition of steroidogenesis continues to prove challenging.

Cytokines play important role in modifying histones and cause significant changes in the pattern of various genes and proteins expression (Halili et al., 2009). Since cytokines mediated inhibition of testicular Leydig cell steroidogenesis occurs mostly at the level of gene expression of different steroidogenic enzymes, it will be valuable to study the testicular histone modifications, in response to endogenous cytokines production. Core histones of chromatin have long N-terminal extensions that have been known for decades to undergo extensive post-translational modifications such as acetylation, methylation and phosphorylation, as well as ubiquitination, sumoylation and ADP-ribosylation (Gallinari et al., 2007). These post-translational modifications have been suggested to be involved in the regulation of
gene expression, cell division, nucleosome assembly, and DNA repair processes via alterations in the nucleosome architecture (Adams and Kamakaka, 1999). To date, acetylation is the best understood of these modifications. Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are two counteracting enzyme families, whose enzymatic activity controls this acetylation process. The classical role of HDACs is to reduce transcription by deacetylation of histone proteins, a process that makes the DNA bind to the histones more tightly of specific lysine residues (Fukuda et al., 2006). HDACs intervene in a multitude of biological processes and are part of a multiprotein family in which each member has its specialized functions.

Class 3 or the sirtuins family of histone deacetylases was named after their homology to the *Saccharomyces cerevisiae* gene silent information regulator 2 (Sir2) and includes 7 (SIRTs 1-7) enzymes. Sirtuins are protein deacetylases/ADP ribosyltransferases that target a wide range of cellular proteins in the nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation (SIRT1, -2, -3 and -5) or ADP ribosylation (SIRT4 and -6). Numerous studies have shown that sirtuins have pathophysiological relevance to neurodegeneration, muscle differentiation, inflammation, obesity, and cancer (Alcendor et al., 2007). Whereas very little is known about the role of sirtuins in regulating signaling pathways involved in reproductive tissues. It has been shown that SIRT1 regulates spermatogenesis at postnatal stages by controlling hypothalamus-pituitary-gonadotropin signaling (Ullas Kolthur-Seetharam et al., 2009). It has also been demonstrated that SIRT2 is essential for normal embryogenesis and normal reproduction in both sexes (Imai and Guarente, 2010). Hence, the present study was aimed at evaluating the effect of bacterial LPS induced inflammation on sirtuins and its implications in testicular Leydig cell function.

This chapter covers the literature review on infections, infections associated inflammation of the male reproductive system, its causes of male subfertility or infertility and role of histone modifications in cellular function.

**1.1 Infertility**

Around 1.2 billion years ago, primitive eukaryotes started to reproduce sexually, which involves the recombination of parental genomes so that a new
genotype is created. This allowed the emergence of more dynamic, adaptable life forms in a shorter period of time. So, sex and fertility are key factors to determine the future of our descendants. Being able to maintain fertility rates above levels of sustained population guarantees the existence of a given population as it is and reduces the risk of extinction, if no other external factors interact (such as a flood, for example). The human species has suffered from variable fertility rates over its history. Nowadays, it is accepted that 2.1 is the minimum fertility rate to guarantee the sustainability of our species (Fig. 1). Until two decades ago, some developing countries could reach a fertility rate of 8, and a general decrease has been noticed since. Postponing marriage and first births, associated with more women pursuing careers, are usually the most accepted reasons for the decrease in fertility rates, which in most Western countries do not even reach the replacement level (Skakkebæk et al., 2006).

**Figure 1.** Fertility rates in 1970 (blue columns) and 2002 (red columns). The minimum fertility rate of 2.1 is also portrayed (green line)

![Fertility rates graph](source)

(Source: Skakkebæk et al., 2006)

Although social behavior can contribute to this change in fertility rates, there are physiological changes that should also be considered, such as falling sperm counts and other reduced sperm quality features (less motility and/or abnormal shape). Plus, the incidence of hypospadias, cryptorchidism and testicular cancer also seem to be increasing (Giwercman et al., 1993; Skakkebæk et al., 2006). With these
great differences in a period of 50-70 years, it seems unlikely that only genetics plays an important role. Instead, changing lifestyle and environmental exposures to hazardous molecules over the years could impair the development of the male reproductive tract and result in a decreased fertility (Skakkebæk et al., 2006).

Infertility is a critical component of reproductive health, and has often been neglected in these efforts (Cui, 2010). The inability to have children affects men and women across the globe. Infertility can lead to distress and depression, as well as discrimination and ostracism (Chachamovich et al., 2010). Infertility is divided into primary and secondary infertility. Definitions of primary infertility vary between studies, but the operational definition, put forth by the WHO, defines primary infertility as the “Inability to conceive within two years of exposure to pregnancy (i.e. - sexually active, non-contracepting, and non-lactating) among women 15 to 49 yr old”. Secondary infertility refers to the inability to conceive following a previous pregnancy. Globally, the incidence of infertility is estimated to be about 13–18% and most infertile couples suffer from primary infertility (Inhorn, 2003).

The recent growth of the Indian population has been unprecedented. It stands currently at over one billion and is expected to touch 2 billion by 2035 (assuming an average growth rate of 2%). Even though curtailting population growth is a major national concern, a substantial number of infertile couples in the Indian population have an equally great concern, that of having a child. This is an equally important national problem concerning reproductive health and the infertile couples have to be treated by medically assisted reproductive technology (MART) for procreation (Seshagiri, 2001). In India according to WHO the overall prevalence of primary infertility ranges between 3.9% and 16.8% (World Health Organization, 2004) and it was reported that the male factor is responsible for 40 per cent. In addition, Statistics indicate that 15 per cent of all couples in the United States and are infertile, and the male factor is responsible for 25 per cent of these cases (Sharlip et al., 2002).

1.1.1 Male infertility

Male infertility refers to the inability of a male to achieve pregnancy in a fertile female. Fifteen percent of all couples in the US are infertile and the male factor is responsible for 25% of these cases (Sharlip et al., 2002). Male factor has been
considered a major contributory factor to infertility with the foremost conventional causes for male infertility are as varicocele, cryptorchidism, obstructive lesions, cystic fibrosis, trauma, tumors, oxidative stress and infections (Agarwal et al., 2008). Men with idiopathic infertility generally present with significantly higher seminal ROS levels, low testosterone and lower antioxidant properties than healthy controls (Pasqualotto et al., 2001).

1. 1. 2 Causes of Male infertility

Male infertility can be caused by problems that affect sperm production, inhibition of steroidogenesis and the sperm transport process. A brief literature review of different causes of male infertility were listed in the below section.

a) Oxidative stress

In recent years, the generation of reactive oxygen species (ROS) in the male reproductive tract has become a real concern because of their potential toxic effects at high levels on sperm quality and Leydig cell function (Aitken and Fisher, 1994). Recent reports have indicated that high levels of ROS are detected in semen samples of 25% to 40% of infertile men (Padron et al., 1997). Oxidative stress (OS) develops as a result of an imbalance between ROS generating and scavenging activities (Sikka, 2001). Spermatozoa are particularly susceptible to Oxidative stress induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes (Agarwal et al., 1997). In addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by depending on the protection afforded by the seminal plasma. Oxidative stress attacks not only the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus (Sikka, 2001).

b) Testicular factors

These are factors that affects quality and quantity of testosterone and semen produced by the testes and include age, genetic defects of the Y-chromosome (Klinefelter syndrome), neoplasm e.g. seminoma, cryptorchidism, varicocele which
account for 14%, mumps viral infection and may be idiopathic which accounts for 30% of male infertility (Olooto et al., 2012).

c) Pre-testicular factors

These are conditions that impede adequate support of the testes and include situations of poor hormonal support and poor general health including hypogonadism; drugs such as cimetidine and spironolactone that decrease follicle stimulating hormone (FSH) levels, and nitrofurantoin that decreases sperm motility; adopted lifestyle (chronic alcoholism, marijuana, cigarette smoking); and strenuous activities such as strenuous bicycle riding decrease sperm motility and testosterone (Leibovitch and Mor, 2005).

d) Hypothalamic-pituitary factors

Conditions that affect the hypothalamo-pituitary-axis will eventually affect the gonadotrophin releasing hormone (GRh) and hence the levels of follicle stimulating hormone, luteinizing hormone and prolactin hormone. These conditions include Kallmann syndrome (isolated gonadotropin deficiency), hyperprolactinemia and hypopituitarism. Hyperprolactinemia may be due to diseases affecting the hypothalamus and pituitary gland or secondary to disease of other organs such as the liver, kidneys and thyroid. Hyperprolactinemia may cause hypogonadism, erectile dysfunction, gynecomastia and infertility (Olooto et al., 2012).

e) Environmental factors

The etiological importance of environmental factors in infertility has been stressed. The implication of toxins such as glues, volatile organic solvents, silicones, physical agents, chemical dusts and pesticides in infertility had been established. Radiations and excessive heat to the genitalia have damaging effect on the testicles. Hence individuals having direct contact with or exposure to such chemicals have high chances of having primary or secondary infertility as the case may be. Estrogen-like hormone-disrupting chemicals such as phthalates are of particular concern for infertility in men and for effects on offspring of women. Exposure to phthalates can occur via dietary consumption, dermal absorption or inhalation and has been linked with impaired spermatogenesis and increased sperm DNA damage. The mechanism for
this is probably due to increase in the generation of reactive oxygen species (ROS) within the testis and a concomitant decrease in antioxidant levels, culminating in impaired spermatogenesis and steroidogenesis as observed in rats (Lee et al., 2007).

f) Life style and infertility

The contribution of tobacco smoking and alcohol intake to infertility was observed to damage sperm DNA. Though some of the damage is irreversible, but stopping smoking can prevent further damage. Smokers are 60% more likely to be infertile than non-smokers. Smoking reduces the chances of IVF producing a live birth by 34% and increases the risk of an IVF pregnancy miscarrying by 30%. Smokers have decreased levels of antioxidants such as Vitamin E and Vitamin C, placing their spermatozoa at additional risk of oxidative damage (Gaur et al., 2007).

g) Sexually transmitted disease (STD) and infertility

STDs are diseases transmitted through sexual activity with an infected partner caused by viruses, bacteria, or parasitic microorganisms. STDs are a leading cause of infertility. They are often asymptomatic but may display few symptoms, with the risk of failing to seek proper treatment in time to prevent decreased fertility. Some of the identified STDs (such as syphilis, trichomoniasis, chancroid, chlamydia, gonorrhea, herpes simplex virus, human papilloma virus, lymphogranuloma venerum) are treatable while many are not and may eventually lead to death (American Society for Reproductive Medicine, 2008).

h) Spermatozoa and infertility

Azoospermia (low sperm counts), abnormal spermatozoa morphology (shape) and low sperm motility, low testosterone are usually asymptomatic conditions to most males but of great etiological importance. Most cases of low sperm counts are "idiopathic" but may be associated with varicocele and chronic testicular diseases. The quality and the total number of spermatozoa are a reflection of the testicular state, plasma testosterone level and the patency of the post-testicular duct system. The total seminal fluid volume is contributed by the various accessory glands and is thus a function of the secretory activity of the accessory glands. The quality of the spermatozoa (count, vitality, motility and morphology) and the composition of seminal
fluid are also important index of sperm function. It is well recognized that sperm DNA can be damaged oxidatively by oxidative stress (Oger et al., 2003) and nonoxidatively by mechanisms such as aberrant apoptosis and incomplete sperm protamination.

**i) Chemotherapy and infertility**

Radiation therapy for cancer can cause damage to the testis, causing permanent problems in steroidogenesis with sperm production. Many young men who have been treated for cancer (for example Hodgkin's disease) with chemotherapy or radiotherapy in the past now have low testosterone or absent sperm production and present to infertility clinics for treatment. Studies have shown that the antral follicle count decreases after the third series of chemotherapy, whereas follicle stimulating hormone (FSH) reaches menopausal levels after the fourth series; inhibin B and anti Mullerian hormone levels decrease following chemotherapy.

Drugs with high risk of infertility include procarbazine, cyclophosphamide, ifosfamide, busulfan, melphalan, chlorambucil and chlormethine; drugs like doxorubicin, cisplatin and carboplatin have medium risk while therapies with plant derivatives (such as vincristine and vinblastine), antibiotics (such as bleomycin and dactinomycin) and antimetabolites (such as methotrexate, mercaptopurine and 5-fluorouracil) have low risk of gonad toxicity (Brydøy et al., 2007).

**j) Microorganisms and infertility**

Microbial infections have been reported to reduce testosterone and sperm viability. Staphylococcus aureus is the most prevalent Gram positive organism, while Escherichia coli is the most prevalent Gram negative organism isolated in the semen of males with primary infertility (Momoh et al., 2007). Chronic epididymitis secondary to Chlamydia trachomatis infection had been shown to blockage of the epididymis and thus obstructive azoospermia. However, urea plasma urealyticum infections induce leukocytospermia and consequently lead to sperm damage, decrease sperm counts and invariably impaired sperm motility. Herpes simplex virus (HSV) was reported to have been found in the semen of some infertile men and was related to low testosterone, sperm count and poor motility. Mumps viral infections in adolescent and
adult males carry about 30% risk of developing orchitis or epididymitis, which can result in testicular atrophy and sterility (Senanayake, 2008).

1.2 Infections, Inflammation and Male infertility

Urogenital infections and inflammation are accepted as significant etiologic factors in male infertility. The percentage of men in andrological populations with infectious and inflammatory entities has been estimated between 6.9 and 8 percent (Agarwal et al., 1997). It is generally accepted that one of the potentially correctable causes of male infertility is symptomatic and asymptomatic infection of the male urogenital tract. Urinary tract infections—urethritis, epididymitis, orchitis, prostatitis and male accessory gland infection have been mentioned in this context to a greater extent in the WHO manual (World Health Organization, 1993; American Society for Reproductive Medicine, 2008). Inflammatory disease has been established as hazard to male reproductive function and fertility.

Infections are associated with disorders in steroidogenesis and spermatogenesis resulting in temporary or permanent male infertility (Agarwal et al., 2012). The significance of localized acute and chronic infections, in particular infections of the male accessory glands, has been considered as causes of male infertility for decades. Infections of the male accessory glands appear to affect reproductive function as indicated by decreased number, density and motility of spermatozoa, and alterations in seminal plasma markers and testosterone biosynthesis (Weidner and Ludwig, 1998). Infections and inflammation account for almost 15% of cases of male infertility seen in infertility clinics (Guazzone et al., 2009).

1.2.1 Inflammation

Bacterial infections are very common type of infections, which vary from mild infections to serious and fatal types of infections. Although the vast majority of bacteria are harmless or beneficial, quite a few bacteria are pathogenic. Inflammation is the body’s normal protective response to infections, injury, irritation, or surgery. It is the part of the complex biological response to vascular tissues to harmful stimuli, such as pathogen, damaged cells, or irritants (Ferrero-Miliani et al., 2007). Inflammation is a protective attempt by the organism to remove the injurious stimuli.
and to initiate the healing process. Inflammation involves activation of monocytes, macrophages and mast cells, leading to production of pro-inflammatory cytokines, prostaglandins and cytotoxic reactive oxygen species, up-regulation of adhesion molecules, recruitment of immune cells, changes in blood flow and increased capillary permeability (Ferrero-Miliani et al., 2007).

Inflammation can be acute or chronic. When it is acute, it occurs as an immediate response to trauma (an injury or surgery) usually within two hours. When it is chronic, the inflammation reflects an ongoing response to a longer-term medical condition, such as arthritis (Cotran, 1998).

a) Acute inflammation

Acute inflammation is a short-term process, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus (Cotran, 1998) and it is characterized by five cardinal signs are pain, heat, redness, swelling, and loss of function (Chandrasoma and Taylor, 2005). The process of acute inflammation is initiated by cells already present in tissues, mainly resident macrophages, dendritic cells, histiocytes, kupffer cells and mastocytes. These cells are provided with certain cell surface receptors named Pattern Recognition Receptors, which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns. The acute inflammatory response requires constant stimulation to be sustained. Inflammatory mediators have short half-lives and are quickly degraded in the tissue. Hence, acute inflammation ceases once the stimulus has been removed (Cotran, 1998).

b) Chronic inflammation

Inflammation that has a slow onset and persists for weeks or more is classified as being chronic. The symptoms are not as severe as with acute inflammation, but the condition is insidious and persistent. The beginning of chronic inflammation is often characterized by the replacement of neutrophils with macrophages and other immune cells, such as T cells (Medzhitov, 2008). During a chronic inflammatory state, granulomas are typically formed in a final attempt to wall off the host from pathogens.
Persistent inflammation leads to increased cellular turnover and provides selection pressure that result in the emergence of cells that are at high risk for cancer. Chronic inflammation is also associated with a variety of cardiovascular, metabolic, and neurodegenerative diseases as well as stroke and myocardial infarction, reproductive disorders (Medzhitov, 2008).

1.3 Bacterial toxin

Many emerging and reemerging bacterial pathogens synthesize toxins that serve as primary virulence factors. These toxins, which affect eukaryotic cells by a variety of means, include Staphylococcus aureus alpha-toxin, Shiga toxin, cytotoxic necrotizing factor type 1, Escherichia coli heat-stable toxin, botulinum and tetanus neurotoxins, and S. aureus toxic-shock syndrome toxin (Schmitt et al., 1999).

1.3.1 Exotoxin

It is classified as a toxin that is released by living bacteria into the environment usually of Gram positive type (streptococcus, staphilococcus) and these are in form of enzymes (hemolysins, coagulases and fibrinolysin). These act on host cells by different mechanisms either by inhibition of cellular protein synthesis or destruction of cell membrane (Schmitt et al., 1999).

1.3.2 Endotoxin

It is considered to be a toxin kept "within" the bacterial cell and to be released only after destruction of the bacterial cell wall. This endotoxin composed of Lipopolysaccharides (LPS): which is a major constituent of the outer cell wall of Gram-negative bacteria (Escherichia coli, Salmonella, Shigella, Klebsiella Pseudomonas, and Neisseria, Haemophilus influenzae, Bordetella pertussis and Vibrio cholerae). The Gram-negative pathogenic bacteria including the “superbugs” A. baumannii, P. aeruginosa and K. pneumoniae can cause severe infections and accounting for over two-thirds of the hundreds of thousands of deaths caused by MDR bacteria in developed economies every year. There are new drugs to treat MDR Gram-positive bacteria but essentially none to treat MDR Gram-negative bacteria (Zhao et al., 2010; Hicks et al., 2013; Wencewicz and Miller, 2013). Infections and inflammation caused by MDR Gram-negative superbug, in particular, P. aeruginosa, are presenting a critical
challenge, and they are associated with a high mortality rate if they are not treated promptly and effectively in hospitalized patients (Cirioni et al., 2008).

**a) Lipopolysaccharide**

Lipopolysaccharide (LPS) is an active component of gram-negative bacteria that activates uncontrolled inflammatory responses. Bacterial Lipopolysaccharide (LPS) is a key virulence factor produced by *E.coli* and plays an important role in mediating the interactions between the bacterium and its host. It has been shown that LPS induces TNF-α and other inflammatory mediators leading to oxidative stress, which in turn induces excessive production of free radicals and antioxidant defenses (Karolina Noworyta-Sokoowska et al., 2013). Imbalance between free radical production and antioxidant defenses is associated with cell damage to a wide range of molecular species including lipids, proteins, and nucleic acids (Lobo et al., 2010). A numerous studies have shown that LPS acutely inhibits Leydig cell function by ROS-mediated disruption of mitochondrial permeability and triggers apoptosis (Allen et al., 2004; Mishra and Dhali, 2007).

**b) Structure of Lipopolysaccharide**

Lipopolysaccharide (LPS) is an essential component of the outer membrane of all Gram-negative bacteria. Structurally, most types of LPS are composed of three distinct regions: the lipid A, the core-oligosaccharide and the O-polysaccharide consisting of long chains of repeating oligosaccharide units (Moran, 2007).

**Region-I:** Lipid A is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Some FA is attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram-negative bacteria. Among Enterobacteriaceae Lipid A is virtually constant. The primary structure of Lipid A and its biological activity appears to depend on a peculiar conformation that is determined by the glucosamine disaccharide, the PO4 groups, the acyl chains, and also the KDO-containing inner core (Raetz et al., 2007).
Region-II: Core (R) antigen or R polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO - Hep - Hep - Glu - Gal - Glu - GluNAc - Two unusual sugars, heptose and 2-keto-3-deoxyoctonoic acid (KDO), are usually present, in the core polysaccharide. KDO is unique and invariably present in LPS and so it has been used as an indicator in assays for LPS (endotoxin).

**Figure 2. Structure of Lipopolysaccharide**

(Source: Lerouge and Vanderleyden, 2002)

Region-III: Somatic (O) antigen or O polysaccharide is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3 - 5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide (Raetz et al., 2007).
c) Mechanism of action

The principal mechanism by which LPS is sensed is via an LPS-binding protein (LBP)–LPS complex and then signalling through the Toll-like receptor 4 (TLR4)–MD-2 complex (Jonathan Cohen, 2002). However, other cell surface molecules also sense LPS; these include the macrophage scavenger receptor (MSR), CD11b/CD18 and ion channels. Intracellular signalling depends on binding of the intracellular TLR domain, TIR (Toll/IL-1 receptor homology domain), to IRAK (IL-1 receptor-associated kinase), a process that is facilitated by two adapter proteins, MyD88 (myeloid differentiation protein 88) and TIRAP (TIR domain-containing adapter protein; also called MyD88-adapter-like protein or Mal), and inhibited by a third protein Tollip (Toll-interacting protein). Note that there is also a MyD88-independent pathway by which TIRAP/Mal signals through an RNA-dependent protein kinase (PKR) and interferon regulatory factor (IRF)-3 (Jonathan Cohen, 2002).

1. 3. 3 Lipopolysaccharide and intra-cellular signaling pathways

LPS is a constituent of the cell wall of Gram-negative bacteria. The major biological activities of LPS are mainly attributed to a lipid component, termed lipid A. LPS interacts with CD14, a receptor on macrophages/monocytes and neutrophils. CD14 is a glycerophosphatidylinositol (GPI)-anchored glycoprotein that lacks a cytoplasmic portion. LPS-binding protein (LBP) might facilitate the interaction of LPS with CD14 (Fenton and Golenbock, 1998) or effect the clearance of LPS from the circulation by transferring LPS from CD14. In certain cell types, such as endothelial cells, that do not express CD14, soluble CD14 (sCD14) substitutes as the signaling bridge for surface recognition of the LPS–LBP complex.

However, at present the molecule that recognizes the complex of LPS–LBP–sCD14 has not been identified in CD142 cells. CD14 does not have a cytoplasmic portion, but substantial evidence supports a role for unidentified signal transducing molecules that interact with CD14 at the cell membrane. Alternatively, binding of LPS to CD14 might result leading to LPS-induced cell activation. Involvement of other protein kinases in the transduction of LPS-induced responses has been also suggested. Such kinases include protein kinase C and mitogen-activated protein...
kinases (MAPK), such as p42 (ERK2), p44 (ERK1) and p38 (Jack et al., 1997; An et al., 2002).

**1. 3. 4 Effect of Lipopolysachharide on various tissues**

**a) Effect of LPS on the brain**

LPS produces changes in the amygdala and hippocampus. It produces alterations in serotonin levels and causes degeneration and reduction in dopamine (DA) neurons. It exacerbates hippocampal damage induced by seizures and affects cerebral blood flow (Nolan et al., 2002).

**b) Effect of LPS on Thyroid gland**

It lowers the thyroid levels but it also reduces thyroid hormone receptor (TR) expression. Furthermore, LPS plays an important part in creating autoimmune thyroiditis as well as goiter. Intravenous administration of low-dose endotoxin (lipopolysaccharide) to healthy subjects reproduced a number of changes in the plasma concentrations of thyroid hormones and TSH commonly seen in NTI, including reduced T4, T3, and TSH concentrations, and increased rT3 levels (Fliers et al., 2015).

**c) Effect of LPS on Kidney**

Renal inflammation is the main pathological change in many acute and chronic kidney diseases (Zhong et al., 2011). Acute renal failure is a frequent complication to the systemic inflammatory response syndrome. LPS will eventually induce widespread endothelial damage with loss of arteriolar tonus in systemic vessels, increased capillary permeability, and sustained hypotension. Furthermore, LPS-induced ARF with deterioration of glomerular filtration rate (GFR) is associated with renal vasoconstriction in the presence of a decrease in the systemic vascular resistance (Jonassen et al., 2002).

**d) Effect of LPS on Testis**

Bacterial lipopolysaccharide (LPS) is a major component of the cell wall of gram negative bacteria contributing to the pathogenesis of bacterial infection, in particular in those diseases affecting reproductive tissues. Infection and inflammation can be reproduced in both *in vitro* and *in vivo* by administration of LPS, and several studies
have observed inhibition of testicular steroidogenesis in animals treated with LPS (O’Bryan et al., 2000; Allen et al., 2004) or with septic agents that generate LPS (Sharma et al., 1996). It has been shown that pro-inflammatory cytokines generated by LPS have an inhibitory role in steroidogenesis through the production of increased reactive oxygen species (ROS) (Reddy et al., 2006).

LPS acutely inhibits Leydig cell function by ROS mediated disruption of mitochondrial permeability (Allen et al., 2004). It has also been demonstrated that LPS treatment is associated with apoptosis of testicular Leydig cells and germ cells (Metukuri et al., 2010; Wu et al., 2012). A numerous studies have shown that LPS plays a pivotal role in the regulation of steroidogenic enzymes gene expression including 3β– hydroxysteroid dehydrogenase (3β–HSD) and 17β– hydroxysteroid dehydrogenase (17β–HSD) (Magata et al., 2014; Sadasivam et al., 2014). However, the exact mechanisms by which LPS causes impaired steroidogenesis and apoptosis are not well studied.

1. 4 Testis as an immune privileged site

1. 4. 1 Structure of the testis

The testes fulfill two major functions for male reproduction: the first is to produce morphological mature and functional spermatozoa, and the second is the production and controlled release of sex steroids (primarily androgens). The testis is compartmentalized both histologically and functionally into two distinct regions to accommodate the two separate functions (Redgrove and McLaughlin, 2014).

a) Leydig cells

Leydig cells produce and secrete the most important male sexual hormone, testosterone. Leydig cells are rich in smooth endoplasmic reticulum and mitochondria with tubular cristae. These physiological characteristics are typical for steroid producing cells and are very similar to those found in other steroidogenic cells, such as those in the adrenal gland and in the ovary (Ge and Hardey, 2007). Other important cytoplasmic components are lipofuscin granules, the final product of endocytosis and lyosomal degradation, and lipid droplets, in which the preliminary stages of testosterone synthesis take place.
**Figure 3.** Mammalian spermatogenesis occurs within the seminiferous tubules of the testis, with development of the mature sperm cells occurring in a radial fashion from the basement membrane into the lumen

![Diagram of spermatogenesis](image)

(Source: Redgrove and McLaughlin, 2014)

Special formations, called Reinke’s crystals, are often found in the adult Leydig cells. These are probably subunits of globular proteins whose functional meaning is not known. The proliferation rate of the Leydig cells in the adult testis is rather low and is influenced by LH. The ontogeny of Leydig cells is not entirely clear and mesonephros, neural crest and coelomic sources have been involved. In the adult testis, Leydig cells develop from perivascular and peritubular mesenchymal-like cells and the differentiation of these cells into Leydig cells is induced by LH but also by growth factors and differentiation factors derived from Sertoli cells (Prince, 2007).

**b) Sertoli cells**

Sertoli cells are somatic cells that provide essential support for developing sperm cells in the testis and have a critical role in the establishment and maintenance
of immune privilege. A number of studies have demonstrated that Sertoli cells possess the ability to act as immune-suppressants and are capable of being transplanted into a variety of tissues and inducing immune tolerance (Li et al., 2010).

c) Blood–testis barrier

The blood–testis barrier is formed by tight junctions between neighboring Sertoli cells localized in the seminiferous epithelium, and function to restrict the movement of molecules through the intracellular spaces at the onset of puberty, developing sperm cells express novel antigens that the immune system would normally identify as “foreign.” However, segregation of antigens in the seminiferous tubules from the immune cells that are able to enter into the interstitial space of the testis prevents the body from eliciting an immune response against these vulnerable cells (Ito et al., 2005).

1. 4. 2 Spermatogenesis

Spermatogenesis is an autonomous process largely under the control of paracrine factors. Growth factors and cytokines that potentially exert effects on cell populations involved in spermatogenesis are produced within the seminiferous epithelium and influence post-meiotic stages of spermatogenesis. Approximately half of the infertility cases are related to men (St. John et al., 2000) and reduced sperm motility is one of the major causes of male infertility just next to reduced count (Ruiz-Pesini et al., 2000; St.John et al., 2000; Spiropoulos et al., 2002a). While the importance of FSH and androgens for the initiation and maintenance of spermatogenesis is clearly documented, the role of paracrine regulatory factors remains to be elucidated. Spermatogenesis is a compartmentalized and continuous process that takes place sequestered within the blood–testis barrier, implicating a need for regulation by locally produced factors (Sharpe, 1994; de Kretser et al., 1998).

Various studies have demonstrated the importance of high local concentrations of androgens to the development of functional spermatozoa (Singh et al., 1995). One potential mechanism subserving inflammatory disease-associated decreases in male fertility is inhibition of testosterone production (Hong et al., 2004).
1. 4. 3 Leydig cells steroidogenesis

Leydig cells are the site of androgen production in the testis. The principal and most important androgen produced by Leydig cells is testosterone. Testosterone biosynthesis is primarily under the control of the pituitary gonadotropin luteinizing hormone (LH). LH stimulates the production of cyclic AMP (cAMP) by binding to specific receptors on the surface of Leydig cells that are coupled to the adenylate cyclase second messenger system. Cyclic AMP is the intracellular second messenger for LH and mediates LH action in Leydig cells by activating the cAMP-dependent protein kinase A (Cooke, 1996).

Figure 4. Leydig Cell Steroidogenesis

(Source: Amaral et al., 2013)
Cyclic AMP has two principal activities in the control of Leydig cell steroidogenesis: The first action of cAMP is the acute stimulation of testosterone biosynthesis via mobilization and transport of cholesterol into the steroidogenic pathway, an action that takes place within minutes. The second action of cAMP in Leydig cells is the chronic and prolonged stimulation of gene expression of the steroidogenic enzymes and up-regulation of their activity, a slower process that requires several hours (Stocco & Clark, 1996a and 1996b). Testosterone biosynthesis is dependent on the action of two cytochrome P450 enzymes and two dehydrogenase enzymes. The first and rate-limiting enzymatic step in testosterone synthesis is the conversion of cholesterol to pregnenolone which is catalysed by the cholesterol side-chain cleavage (P450scc) enzyme that is located on the inner mitochondrial membrane. Pregnenolone diffuses out of the mitochondria to the smooth endoplasmic reticulum where it is further metabolized via the action of 3β-hydroxysteroid dehydrogenase D4–D5- isomerase (3β-HSD) to progesterone. Progesterone in turn is converted by a two-step process to androstenedione via the action of 17α-hydroxylase/C17-20 lyase (P450c17). The conversion of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) (Payne and O’Shaughnessy, 1996).

**1.5 Cytokines**

Immunological events include the migration of white blood cells to the site of infection due to chemotaxis and the secretion of cytokines that are necessary for the communication between immune competent cells. Besides this function, cytokines exert a variety of effects on tissues and cells not primarily involved in the immune defense. In fact, cytokines appear to be responsible for most pathophysiological events associated with infections and are the decisive factors that determinate the pathology of an infectious disease. It has long been appreciated that chronic inflammation and systemic infection are associated with decreased reproductive capacity (Handelsman, 1994). In addition, cytokines have decisive activities outside of the immune system where they function as regulators of testicular steroid hormone production. Cytokines also have been implicated as novel growth and differentiation factors involved in the regulation of cell in both the endocrine and the tubular compartment of the testis. Men with critical illness, burn trauma, sepsis and rheumatoid arthritis are reported to
have markedly reduced serum testosterone levels resulting in infertility (Handelsman, 1994).

1. 5. 1 Cytokines and Steroidogenesis

Cytokines interact and modulate steroidogenesis at the levels of the adrenal glands, testes, and ovaries, influencing function and development of these glands in a complex and systemic manner. Within the HPA axis cytokines directly and/or indirectly act on production and secretion of cortisol and ACTH, where adrenal steroidogenesis in general is mainly affected by locally (intraadrenal) produced cytokines. For example, maintenance of chronic stress can partly be explained by the action of cytokines leading to an up-regulation of adrenal steroidogenesis. On the other hand, growth and differentiation of the fetal adrenal is affected by cytokines, and cytokines also act on adrenal tumorigenesis. Cytokines, as e.g. IL-1, IL-6, TNF-α, and LIF, are key players in the immune-adrenocortical communication, can have tumorigenic properties (e.g. IL-8) and directly modulate adrenal function (Bornstein et al., 2004).

On the level of the testes, cytokines interfere with proliferation of immature Leydig cells and are able to inhibit adult Leydig cell steroidogenesis and testosterone production. Especially IL-6 has been shown to influence the hypothalamic–pituitary–testicular axis in men, showing adverse effects on male reproductive function, suppressing Leydig cell steroidogenesis and testosterone levels in healthy men. Within the hypothalamic–pituitary–ovarian axis cytokines, as e.g. IL-8 and MCP-1 interfere with follicular development and atresia, ovulation, steroidogenesis, and corpus luteum function. Other cytokines, as IL-6, co-regulate the ovarian steroid production, whereas some ovarian cancer cells secrete cytokines as TNF-α and IL-1 which stimulates growth of these cells (Willenberg et al., 2002).

During stress there is an activation of the adrenal whereas gonadal steroidogenesis is inhibited. This is part of a physiological fight and flight reaction and cytokines seem to play an important role in mediating these differential effects on the steroid producing cells particularly during infection, inflammation, and sepsis. During chronic stress high levels of inflammatory cytokines may be necessary to maintain higher glucocorticoid levels allowing a shift away from androgen and estrogen
synthesis towards increased cortisol production. Whereas this may be beneficial during sepsis or other severe illnesses it may also contribute to a higher risk profile in patients with diabetes, depression, and autoimmune diseases. Finally, continuous exposure to elevated cytokine levels in conjunction with increased or aberrant receptor expression may lead to uncontrolled stimulation and proliferation of steroidogenic tissue and contribute to tumor formation in the adrenals, ovaries, and testes. Particularly in the light of new cytokine antagonists as a promising treatment for rheumatoid arthritis and other autoimmune disorders elucidating the complex immune-endocrine interactions will have high priority (Bornstein et al., 2004).

**a) Tumor Necrosis Factor-α**

There are two biologically active forms of TNF-α: the full-length type II transmembrane protein and the soluble form resulting from proteolytic cleavage of transmembrane TNF-α by TNF-α-converting enzyme (Black, 2002). Transmembrane and soluble TNF-α exert their effects by binding, as a trimmer, to either cell membrane TNF-α receptor (TNFR) TNFR1 or TNFR2. TNFR1 contains a cytoplasmatic death domain and belongs to the family of death receptors responsible for the transduction of TNF-α induced death signal through caspase activation; survival and proinflammatory signals may also be initiated by stimulation of TNFR1. This type of signal predominates depends on the balance of intracellular adaptor proteins interacting with TNFR1. Because the intracellular domain of TNFR2 lacks death domain, it efficiently activates NF-kB pathway and JNK and is generally unable to elicit apoptosis (Dempsey et al., 2003).

Different interstitial cells such as mast cells (Rodriguez et al., 2006), lymphocytes and macrophages synthesize TNF-α (Bryniarski et al., 2005). Testicular macrophages released more TNF-α when stimulated by LPS highlighting the ability of these cells to modify the normal immunosuppress or microenvironment of the testis (Hales et al., 1999). TNF-α is a multifunctional cytokine involved in spermatogenesis through the modulation of androgen receptor expression in Sertoli cells, transport of iron to germ cells, and lactate supply to postmeiotic germ cell and spermiation (Lysiak, 2004). TNF-α modulates testicular germ cell apoptosis and survival through the regulation of Fas/Fas L system (Riccioli et al., 1995; Pentikäinen et al., 2001). A direct effect of TNF-α on germ cell survival mediated by the upregulation of Bcl-xL, a
prosurvival protein of the Bcl-2 family protein, was also demonstrated (Suominen et al., 2004). On the other hand, TNF-α is able to induce the expression of proinflammatory mediators in the testis. In combination with other cytokines, TNF-α stimulates in-vitro rat Sertoli cells to express inducible NOS and the release of NO (Bauché et al., 1998). TNF-α induces the expression of intracellular adhesion molecule-1, vascular adhesion molecule (VCAM-1), and IL-6 on cultured Sertoli cells and VCAM-1 and IL-6 on peritubular cells, suggesting that this cytokine may induce the migration of lymphocytes from vasculature to interstitium (Riccioli et al., 1995; Schell et al., 2008).

b) Interleukin-6

IL-6 is one of the most potent cytokines that promote inflammatory events through expansion and activation of T cells, differentiation of B cells, and induction of the acute phase response. IL-6 actions are mediated by IL-6R, a glycoprotein of 80 kDa (gp80), considering to be the α-subunit of IL-6R. Binding of IL-6 to IL-6R leads to the homodimerization of gp130, considered to be the β-subunit of IL-6R, and activation of the signal transduction pathways, which generates opposite responses such as cell growth and differentiation or growth arrest and apoptosis (Kamimura et al., 2003).

In normal rats, IL-6 is produced by most of testicular somatic cells: interstitial macrophages, Leydig cells, Sertoli cells, peritubular, and germ cells (Potashnik et al., 2005; Rival et al., 2006b). In-vitro experiments showed that IL-6 induced germ cell apoptosis when added to the cultures of rat seminiferous tubules. In addition, the apoptotic effect of IL-6 on germ cells may be mediated through the modulated expression of pro- or antiapoptotic factors or through the inhibition of meiotic DNA synthesis as it was demonstrated in preleptotene spermatocytes and, to a lesser extent, in advanced spermatogonia in the normal testis (Rival et al., 2006b).

1. 6 Transcriptional Regulation of steroidogenesis

Expression of the genes that mediate the first steps in steroidogenesis, the steroidogenic acute regulatory protein (STAR), the cholesterol side-chain cleavage enzyme, cytochrome P450scc (CYP11A1) and 3β-hydroxysteroid dehydrogenase/ D5-D4 isomerase (HSD3B), is tightly controlled by a battery of transcription factors in the
adrenal cortex, the gonads and the placenta. (Fayard et al., 2004). These genes generally respond to the same hormones that stimulate steroid production through common pathways such as cAMP signaling and common actions on their promoters by proteins such as NR5A and GATA family members. However, there are distinct temporal, tissue and species-specific differences in expression between the genes that are defined by combinatorial regulation and unique promoter elements (Sugawara et al., 1997).

**a) Steroidogenic Factor 1 (SF-1) and Liver Receptor Homologue 1 (LRH-1) Family**

The orphan nuclear receptors SF-1 (NR5A1) and LRH-1 (NR5A2) are present in all steroidogenic cells in the gonads and adrenal. The two proteins bind the same DNA sequence (Fayard et al., 2004) and regulate critical genes in the reproductive axis and steroidogenesis (Caron et al., 1997), including STARD1. Multiple conserved NR5A recognition sequences have been identified in the human STARD1 promoter with at least two functional elements at _105 to _95 and _42 to _35 and one at _926 to _918, relative to the transcriptional start site (Sugawara et al., 1997). So far, LRH-1 has been shown to activate several promoters just as well as SF-1, including StAR, Cyp17, P450sc, Cyp19, HSD3B2, and INSL3 (Hiroi et al., 2004). Therefore, it is likely that LRH-1 and SF-1 could play redundant roles in the transcriptional regulation of several genes in Leydig cell. A definitive answer as to the in-vivo contribution of LRH-1 to Leydig cell function remains unknown, as LRH-1−/− mice die in-utero at embryonic day 6.5 as a result of severe gastrulation defects (Lopez et al., 2001).

Upon hCG treatment, LRH-1 associates with the endogenous STARD1 promoter in ovarian tissue in mice. Targeted loss of LRH-1 in granulosa cells also disrupts STARD1 mRNA levels and progesterone production in hCG-treated transgenic mice (Duggavathi et al., 2008; Yivgi-Ohana et al., 2009). Venteclef et al. [2006] have shown that a substantial reduction of LRH-1 expression with a parallel induction of TNF-α in LPS treated hepatocytes. Moreover, it has been confirmed that LRH-1 upregulates the expression of the steroidogenic enzymes (Mueller et al., 2006). While SF-1 and LRH-1 have important functions in regulating STARD1 gene expression, their precise roles and sites of action may require further revision given recent findings regarding a second orphan nuclear receptor family, NR4A.
b) NR4A Family

NR4A transcription factors represented by Nur77 (NGFI-B; NR4A1), nur related protein-1 (Nurr1; NR4A2) and neuron-derived orphan receptor 1 (Nor1; NR4A3) bind sequences similar to SF-1/LRH-1 sites, called NGFI-B response elements (NBREs) (Hsu et al., 2004). LH also up regulates Nur77 in primary rat Leydig and MA-10 and K28 mouse Leydig tumor cells (Martin et al., 2008; Song et al., 2001). Nur77 is the predominant NR4A protein in steroidogenic cells and its expression activates the murine STARD1 promoter to various degrees in Y1 and Leydig tumor cells. It recognizes the proximal NRBE, which coincides with the _105 to _95 SF-1 site, and cAMP stimulation increases its association with the region of the STARD1 promoter containing this element in Leydig cells (Manna et al., 2009; Havelock et al., 2005).

c) GATA Family

The role of GATA factors is very significant. The GATA site located at _63 to _58 in the human and _66 to _61 in the mouse is highly conserved. This region efficiently binds recombinant GATA4 and GATA6. The site comprises about 20% of the cAMP responsiveness of the murine promoter in MA-10 Leydig cells (Wooton-Kee and Clark, 2000) and 30–35% of the maximal FSH response in cultured granulosa cells. Accordingly, association of GATA4 with the endogenous proximal promoter in MA-10 cells increases within 15 minutes of treatment with 8-bromo-cAMP. While both overexpressed GATA4 and GATA6 effectively drive porcine STARD1 promoter activity in granulosa cells, which GATA protein actually directs, STARD1 expression may vary with the level of differentiation (Gillio-Meina et al., 2003).

1. 7 Intracellular signaling pathways of steroidogenesis

To function adequately, Leydig cells require the action of pituitary LH. As shown in Fig. 5, LH binds to its receptor, LHCGR, located on the surface of Leydig cells, which triggers activation of adenylate cyclase leading to increased cAMP production. This in turn activates of several kinases, the best studied being protein kinase A (PKA) and PKC (Smith and Walker, 2015). Another important contributor to LH-induced steroidogenesis is the epidermal growth factor receptor (EGFR). Indeed, inhibition of EGFR signaling blunts LH-induced steroidogenesis in Leydig cells (Jamnongjit et al.,
2005). It was later reported that activation of LHCGGR leads to a transient cAMP-dependent activation of the EGFR and downstream mitogen-activated protein kinase (MAPK) cascade (Evaul and Hammes, 2008). The involvement of the MAP kinases extracellular signal-regulated kinases 1 and 2 (ERK1/2) downstream of PKA have long been known to be essential for proper LH-induced steroidogenesis in Leydig cells (Matzkin et al., 2013; Yamashita et al., 2011). The cGMP signaling pathway was also found to stimulate steroidogenesis. cGMP is produced by the enzyme guanylate cyclase (GC) following exposure of Leydig cells to natriuretic peptides (ANP and CNP) or to nitric oxide.

**Figure 5. Intracellular signaling pathways of Testosterone production**

(Source: Tremblay, 2015)
The cGMP pathway was found to act mainly via the protein kinase G at the level of the STAR protein to increase basal steroid hormone production in Leydig cells. In addition to the increase in intracellular cAMP/cGMP levels, a transient increase in cytoplasmic Ca2+ concentration following tropic hormone stimulation is also essential for proper steroidogenesis (Andric et al., 2007). Murine Leydig cells contain two main L-type Ca2+ channel receptors: the ryanodine receptors I, II, and III, and the inositol triphosphate receptors I, II, and III (Costa et al., 2010). While both types of receptors are required for the LH induced rise in cytoplasmic Ca2+ levels, the Ca2+ influx was found to originate mainly from activation of the ryanodine receptors located on the endoplasmic reticulum (Abdou et al., 2013). Increased intracellular Ca2+ concentration activates the Ca2+/calmodulin kinase kinase (CaMKK) pathway, which in turn leads to activation of the Ca2+/calmodulin kinase I (CAMKI). CAMKI was found to be present in Leydig cells, activated downstream of cAMP following hormonal stimulation, and essential for maximal hormone responsiveness (Martin et al., 2008; Martin et al., 2009). These data expand the scope of cAMP action in Leydig cells and provide alternate signaling routes leading to proper gene expression in response to cAMP.

Leydig cell steroidogenesis is mainly regulated by LH through its G protein-coupled receptor, which then activates various signaling pathways. To ensure a proper physiological output, these signaling pathways must be converted into a genomic response in order to appropriately modulate expression of genes involved in testosterone biosynthesis. One of the best studied LH-responsive gene in Leydig cells is the steroidogenic acute regulatory protein (StAR), which codes for a protein implicated in the process of cholesterol shuttling from the outer to the inner mitochondrial membrane, an essential step for the initiation of steroidogenesis. The StAR gene is expressed at low levels in the absence of a stimulus and its expression is rapidly induced in response to hormonal stimulation (Manna et al., 2009). Star gene expression was found to involve several transcription factors (King and Lavoie, 2012), which can be divided into two groups: those already present in the cell which are activated by post-translational modifications, and those that are de novo synthesized (Martin et al., 2008).
As shown in Fig. 5, the transcription factors already present in the cell include SF1 (NR5A1, Ad4BP), LRH1 (NR5A2), COUP-TFII (NR2F2), GATA4, C/EBPb, SP1, CREB/CREM, members of the AP1 family (cFOS and cJUN), and MEF2. All these factors were shown to stimulate StAR gene expression and thus steroidogenesis in cells. Although all are involved in basal Star transcription, some also participate in the hormone-mediated increase in Star expression following their activation mainly by phosphorylation (Manna et al., 2009; King and Lavoie, 2012). The atypical nuclear receptors DAX1 (NR0B2) and SHP (NR0B2) were shown to repress the expression of several steroidogenic genes thus inhibiting steroidogenesis in Leydig cells (Ahn et al., 2013). DAX1 is associated with co-repressors such as NCoR while SHP primarily acts by preventing co-activator recruitment. Both DAX-1 and SHP physically interact with DNA-bound transcriptional activators such as SF1, NUR77 and LRH1 thus repressing their activity (Song et al., 2004).

A similar mechanism of action was described for the leucine rich protein ARR19 (androgen receptor co-repressor of 19 kDa), a known repressor of steroidogenesis. ARR19-mediated inhibition of steroidogenic gene expression is caused by interference in the recruitment of the co-activator SRC1 to NUR77 (Qamar et al., 2010). NUR77 is known to cooperate with the SRC co-activators to enhance steroidogenic gene expression in Leydig cells (Martin and Tremblay, 2005). Interestingly, ARR19 expression is down-regulated in response to LH/cAMP, and as such contributes to the hormone-induced increase in steroidogenesis.

In response to hormonal stimulation, Leydig cell steroidogenesis increases and this is associated with an up regulation of genes involved in this process, including StAR. As Fig.5 mentioned above, maximal hormone responsiveness of StAR gene expression and steroidogenesis requires newly synthesized transcription factors (Martin et al., 2009). To date, the expression of a handful of transcription factors, known to be involved in steroidogenesis, is increased in response to LH/hCG/cAMP signaling in Leydig cells. These include C/EBPb (about 3-fold), cJUN (about 6-fold), and NUR77 (about 30-fold). Furthermore, cooperation between transcription factors also contributes to the overall increase in steroidogenesis in response to hormonal stimulation. For instance, interactions between GATA4 and C/EBPb, CREB and SF1, SF1 and C/EBPb, SP1 and SF1, MEF2 and GATA4, DLX5/6 and GATA4, and between
cJUN and SF1, GATA4, and C/EBPb (Martin et al., 2012; Martin et al., 2009) have been reported on the StAR promoter.

1.8 Homeostasis of testosterone levels

Once testosterone released in the bloodstream has reached a sufficiently high level, it acts on the pituitary and hypothalamus via the negative feedback loop to suppress GnRH and LH production, which will in turn lead to a passive reduction in testosterone production by Leydig cells. For instance, expression of the genes encoding the MAPK phosphatases 1 and 2 (MKP1, MKP2) is induced in response to LH/hCG. In Leydig cell, MKP1 and MKP2 were found to dephosphorylate ERK1/2 leading to their inactivation and resulting in reduced expression of steroidogenic genes (Gomez et al., 2013; Brion et al., 2011). Another mechanism involves the phosphodiesterase (PDE) enzymes present in Leydig cells, mainly PDE4, PDE5, PDE8A, and PDE8B. These enzymes reduce the response to LH by converting the newly synthesized cAMP/cGMP into AMP/ GMP (Andric et al., 2010). As a consequence, the steroidogenic output is also decreased (Shimizu-Albergine et al., 2012).

Recent studies have revealed the existence of an active repression mechanism within the Leydig cell itself, which involves the AMP-activated kinase (AMPK). This kinase is activated by the high AMP levels present in Leydig cells (Fig. 5), which occur following cAMP degradation by the PDEs. Once activated, AMPK was found to rapidly lower hormone-induced steroid production by two complementary mechanisms. First, activated-AMPK represses hormone-induced expression of StAR and of two of its activators, cJun and Nur77. Second, AMPK also acts by increasing the expression of two repressors of steroidogenesis, cFos and Dax1 (Abdou et al., 2014). AMPK acts as an energy sensor of the cell and works as a key regulator of mitochondrial biogenesis (Jornayva and Shulman, 2010).

1.9 Mitochondria energy metabolism and inflammation

Inflammation is an evolutionarily conserved, coordinated response to harmful stimuli, with a goal of returning to homeostasis (Medzhitov, 2010). Thus, the inflammatory response has a unifying purpose encoded in the germline: protection and restoration. Emerging data support that switches in bioenergy inextricably link metabolism with inflammation and immunity to protect cells and organisms and to
restore homeostasis (Gillum et al., 2011). As a result, metabolic polarity exists between the anabolic pro-inflammatory phase, which requires glycolysis to meet the rapid demands for high energy during the early response to a threat, and the catabolic adaptation phase, which depends on fatty acid oxidation to heal and restore homeostasis (Zhong and Mostoslavsky, 2010; Verdin et al., 2010). The adaptation phase has been variously called a compensatory anti-inflammatory response or endotoxin tolerance, but the term “adaptation phase” better reflects this complex reprogramming state for inflammatory and metabolic signaling pathways and genes. During acute systemic inflammatory diseases, such as sepsis, the polarity is sequential and predictable, shifting from the pro-inflammatory phase to the adaptation phase, whereas in chronic inflammatory diseases, such as diabetes, obesity with metabolic syndrome, and atherosclerosis, the pro-inflammatory phase dominates and persists, unless there are external changes in nutrition and bioenergy requirements (McCall et al., 2011). Importantly, the adaptation phase of acute systemic inflammation from sepsis is associated with immunosuppression of innate and antigen-specific acquired immunity. The interplay of cellular bioenergetics, metabolism, and inflammation occurs in innate and adaptive immune responses associated with inflammatory diseases, and similar changes may occur in organ-specific tissues (McCall et al., 2011).

Bioenergy balance between AMP and ATP and oxidized NAD+ and reduced NADH inform many cellular functions associated with inflammation and metabolism, including intracellular signaling pathways, nuclear transcription factors, and chromatin structure (Galli et al., 2010). Increases in ATP production and NADH formation decrease the ratios of AMP/ATP, and NAD+/NADH occurs during the early proinflammatory-phase response of innate and adaptive immunity effector responses. During the switch from the pro-inflammatory and pro-immune phase to the adaptation phase, elevated ratios of AMP/ATP and NAD+/NADH and/or the de novo generation of NAD+ become pivotal regulators of cellular metabolism.

In animals, NAD+ production is controlled primarily by the rate-limiting enzyme, Nampt, which is expressed as iNampt and eNampt (Galli et al., 2010), induced by AMP sensor AMPK (Rodgers et al., 2005). Surprisingly eNampt has proinflammatory-phase properties and provides an extracellular source of NAD+ (Yang
et al., 2006). Important features of NAD+ biosynthesis, in addition to generating NAD+, include NMN production and activation of NMNAT, which occurs in three forms that translocate in distinct regions within cells (nucleus, Golgi complex, and mitochondria) to provide compartment-specific production of NAD+ (Imai and Guarente, 2010). Thus, growing evidence indicates that the proinflammatory-phase immunocytes of chronic inflammation are “suspended” in a glycolytic and low mitochondrial glucose oxidation state, accompanied by elevated ROS and dysregulated mitochondrial biogenesis (Gillum et al., 2011).

1. 10 Mitochondrial Adenine Nucleotide translocator (ANT)

The free radical theory of aging proposes that reactive oxygen species (ROS) play a causative role in the inflammation process, and supports the mitochondrial theory of aging, because mitochondria produce oxygen radicals while they produce cellular ATP (Belzacq et al., 2001). Furthermore, mitochondrial functional decline is closely associated with the infections related accumulations of oxidative stress, an accelerated rate of mitochondrial DNA mutation, and energy depletion. Many mitochondrial proteins are encoded by nuclear and mitochondrial DNA to produce ATP and to regulate cellular metabolism. Therefore, alterations in mitochondrial protein components can critically diminish mitochondrial function and lead to male infertility, which is known to be associated with infections and the effects of reproductive diseases (Byrne et., 1999).

Of the thousands of proteins in mitochondria, adenine nucleotide translocator (ANT) plays an important role in mitochondrial bioenergetics by functioning as an antiporter localized in the inner mitochondrial membrane to replace matrix ATP with cytosolic ADP and provide the cytosol with newly synthesized energy. Therefore, ANT importantly maintains oxidative phosphorylation and mitochondrial membrane potential. Moreover, ANT also regulates permeability transition pore complex, which contributes to mitochondria-mediated apoptosis (Galganska et al., 2010).

It is known that the mouse possesses two primary ANT isoforms, ANT1 and ANT2, and a testis isoform, ANT4. Mouse ANT1 is expressed in skeletal muscles and in the heart and brain. On the other hand, ANT2 is highly expressed in proliferating and regenerating cells or tissues, such as, lymphocytes, the kidneys and liver (Hyde et al.,
In addition to its bioenergetic functions, ANT1 has been documented to have a pro-apoptotic function (Jang et al., 2008). However, the functional role of the more ubiquitously expressed ANT2 has received little research attention.

1. Mitochondria and Male Reproductive function

Mitochondria and its role in male reproduction has remained an enigma since long. Similarly, etiology of male infertility in a large percentage of individuals, mainly primary infertility, has evaded concrete conclusions. Oxidative metabolism, energy production and free radical generation are the principal biological reactions occurring inside mitochondria. In addition to the above, mitochondria participates in an important process of testicular steroidogenesis and apoptosis. Mitochondrial causes of infertility have triggered interest because of its presence in the tail of sperm and immense need of energy for sperm motility and testosterone biosynthesis. Several studies on mitochondria have strongly suggested its role in fertility, some of which support mitochondrial role presenting numerous hypotheses, whereas others deny its very existence as a causative factor (Larsson et al., 1996).

Mitochondrion was first correlated with male infertility in the early 1990s when researchers (Folgerø et al., 1993) reported reduced sperm motility and testosterone in the individuals having structural defects in mitochondria. In mammalian germ cells, ROS production has been shown to be an essential physiological event for maturation, capacitation and acrosomal reaction of spermatozoa, binding with zona pellucida and oocyte fusion. Excessive generation of ROS has been found to be associated with idiopathic male infertility (Said et al., 2005), Leydig cell and sperm apoptosis (Gandini et al., 2000), impaired pre-implantation and development of the embryo and increased rate of early pregnancy loss. Two ROS generating systems have been proposed in sperm: nicotinamide-adenine dinucleotide phosphate oxidase, located in the plasma membrane and nicotinamide-adenine dinucleotide oxidase-dependent oxidoreductase (diaphorase), located in the mid-piece of spermatozoa and integrated in the mitochondrial respiratory system (Said et al., 2005). The sperm plasma membrane has very high concentrations of polyunsaturated fatty acids and it has no membrane repairing capacity, making it a soft target for ROS-mediated damage. In addition, High physiological activity in testis results in ROS generation, which may impair Leydig cell function through lipid peroxide, induced changes in membrane fluidity and integrity,
loss of the membrane-bound adenosine triphosphatase and adenosine triphosphate depletion. At this level, it becomes difficult to answer which mutations and injuries are tolerated or removed by apoptosis, i.e., whether nuclear induced or inherent in mitochondria and how they are related (Larsson et al., 1996). Nevertheless, it is clear that mitochondria are liable to pay the penalty for harbouring the principle ROS generating machinery.

Adrenal, gonadal, placental and brain mitochondria contain several steroidogenic enzymes, notably the cholesterol side chain cleavage enzyme, P450scc, which is the enzymatic rate-limiting step in steroidogenesis which determines cellular steroidogenic capacity (Miller, 2011). Mitochondria play highly specialized, indispensable roles in the production of steroid hormones, which are necessary for life-sustaining homeostasis and reproduction in all vertebrates. Cytokines play important role in modifying histones and cause significant changes in the pattern of various genes and proteins expression (Halili et al., 2009).

The expression of steroidogenic enzymes genes is regulated mainly at the transcriptional level by histone associated enzymes (Hiroi et al., 2004). Since the early reactions of testicular steroidogenesis occur in mitochondria, we speculate histone associated enzymes may have a role in regulation of steroidogenesis.

1. 12 Epigenetics

Epigenetics is defined as the study of mitotically and meiotically heritable changes in gene function that are not dependent on DNA sequence (Feinberg, 2007). The molecular basis of epigenetic processes is complex and involves modifications of histones, methylation of DNA, positioning of histone variants, and gene regulation by non-coding RNAs. The epigenome, the overall epigenetic state of an organism, is just as important as the genome to normal development. Importantly, environmental factors (nutrients, toxins, infections, hypoxia) can have profound effects on the epigenetic signature and trigger susceptibility to disease (Barros and Offenbacher, 2009; Safronova and Morita, 2010). Epigenetic modifications are potentially reversible, and, therefore, a thorough understanding of these changes may identify new therapeutic targets for disease.
1. 12. 1 **Histone modifications**

In all eukaryotes, chromatin is a highly condensed structure that forms the scaffold of fundamental nuclear processes such as transcription, replication and DNA repair (Marmorstein, 2001). Chromatin exists in at least two conceptually distinct functional forms: a condensed form during mitosis and meiosis that generally lacks DNA regulatory activity, called heterochromatin; and a looser de-condensed form, which provides the environment for DNA regulatory processes, called euchromatin.

Nucleosomes are the building blocks of chromatin and they represent two turns of genomic DNA (147 base pairs) wrapped around an octamer of two subunits of each of the core histones H2A, H2B, H3, and H4. The amino-terminal portion of the core histone proteins contains a flexible and highly basic tail region, which is conserved across various species and is subject to various post-translational modifications. The structure of chromatin fulfills essential functions, not only by condensing and protecting DNA, but also in preserving genetic information and controlling gene expression (Safronova and Morita, 2010). Core histones of chromatin have long N-terminal extensions that have been known for decades to undergo extensive post-translational modifications such as phosphorylation, sumoylation and ubiquitination, methylation, as well as ADP-ribosylation, and acetylation.

**a) Phosphorylation**

Protein phosphorylation represents the addition of a phosphate (PO4) group to a protein molecule. Phosphorylation is catalyzed by various specific protein kinases, whereas phosphatases mediate removal of the phosphate group. Multiple serine and threonine residues of histones are subject to phosphorylation by a wide array of kinases (Baek, 2011). Since the intracellular ATP concentration is well above the Km of most kinases, metabolism should not exert a direct effect on histone phosphorylation.

However, some kinases that are responsive to metabolic changes can phosphorylate histones, thus providing an indirect way for metabolic control of histone phosphorylation. For example, in response to a low ATP/AMP ratio indicative of metabolic stress, AMPK can translocate to chromatin and phosphorylate histone H2B...
serine 36. The H2BS36 phosphorylation facilitates the expression of AMPK dependent genes and is essential for metabolic adaptation and cell survival under energetic stress (Bungard et al., 2010).

b) **Sumoylation**

Sumoylation consists in the addition of a “Small Ubiquitin-related modifier protein” (SUMO) of ~100 amino acids. Similar to ubiquitination, SUMO is always covalently attached to other proteins through the activities of members of an enzymatic cascade (E1-E2-E3). Histone sumoylation was first reported in 2003, when Shiio et al. found that H4 can be modified by SUMO and they suggested that this modification leads to the repression of transcriptional activity through the recruitment of HDACs and HP1 proteins. Histone sumoylation has a role in transcription repression by opposing other active marks such as acetylation and ubiquitination (Shiio and Eisenman, 2003).

c) **Ubiquitination**

Ubiquitin is a 76 amino acid protein highly conserved in eukaryotes. Ubiquitination (or ubiquitylation) refers to the post-translational modification of the amino group of a lysine residue by the covalent attachment of one (monoubiquitination) or more (polyubiquitination) ubiquitin monomers. Typically, polyubiquitination marks a protein to be degraded via the 26S proteasome, whereas monoubiquitination modifies protein function. The immune system must operate in an effective, precise and safe manner to defend against diverse pathogens while avoiding attacking the body itself and commensal bacteria. Inflammatory pathways mediated by NOD-like, Toll-like, RIG-I-like and tumor-necrosis-factor receptor families are tightly regulated by ubiquitination, especially by Lys63-linked and linear polyubiquitin chains (Corn and Vucic, 2014).

d) **Methylation**

DNA methylation is the covalent transfer of a methyl group from S-adenosyl-L-methionine to cytosines in CpG dinucleotides. DNA methylation is important in the regulation of inflammatory genes. Promoter hypo methylation of the Toll-like receptor
2 (TLR2) gene is associated with increased pro-inflammatory response to bacterial peptidoglycan in cystic fibrosis bronchial epithelial cells (Shuto et al., 2006). DNA methylation and histone acetylation regulate TLR4 in intestinal epithelial cells (Takahashi et al., 2009). DNA methylation and histone modifications play an important role in the establishment of the epigenetic landscape across the TNFα locus (Sullivan et al., 2007).

e) ADP-ribosylation

In eukaryotic cells, nicotinamide adenine dinucleotide (NAD+) is a key metabolite that participates in a vast number of redox reactions but is also involved in a variety of signaling reactions. Apart from being used as substrate for the formation of several metabolites, including free nicotinamide, ADP-ribose, O-acetyl-ADP-ribose (OAADPr), and cyclic ADP-ribose, NAD+ has also an essential role in the modification of proteins. ADP-ribosylation is a reversible posttranslational modification (PTM) that results from the transfer of the ADP-ribose moiety from NAD+ to specific amino acid residues on substrate proteins or to ADP-ribose itself. The reaction is catalyzed primarily by various ADP-ribosyltransferases (ARTs) and a subgroup of NAD+-dependent sirtuins (Hottiger et al., 2010; Houtkooper et al., 2012).

ADP-ribosylation is conserved in all organisms from bacteria to humans, except in yeasts. It was initially discovered when the addition of NAD+ to hen liver nuclear extracts was found to stimulate synthesis of poly-ADP-ribose (PAR) (Ha and Snyder, 1999). In cells, the involvement of NAD+ in PAR formation requires constant resynthesis of NAD+ to avoid depletion of the intracellular NAD+ pool. Intracellular NAD+ levels decrease by only 5–10% upon moderate PAR synthesis, but severe or sustained stress-induced PAR synthesis results in near-complete depletion of the cellular NAD+ pool, eventually leading to necrotic cell death due to ATP depletion and an increase of AMP in an attempt to counteract this loss (Andrabi et al., 2014). Thus, it is likely that NAD+ production is strictly controlled under normal physiological conditions by biosynthetic enzymes at sites of NAD+ demand.

f) Acetylation

Post-translational modifications have been suggested to be involved in the regulation of gene expression, cell division, nucleosome assembly, and DNA repair
processes via alterations in the nucleosome architecture (Adams et al., 1999). To date, acetylation is the best understood of these modifications. Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are two counteracting enzyme families, whose enzymatic activity controls this acetylation process (Fig. 6).

**Figure 6.** Role of Histone acetylation/deacetylation in regulation of gene expression

![Figure 6](source)

(Source: Kurdistani and Grunstein., 2003)

**Table 1.1. Classifications of histone deacetylases**

<table>
<thead>
<tr>
<th>Class</th>
<th>Homology in yeast</th>
<th>Enzyme</th>
<th>Protein length (amino acids)</th>
<th>Catalytic domains</th>
<th>Mechanism</th>
<th>Subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rpd3</td>
<td>HDAC 1</td>
<td>482</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>Nuclear</td>
</tr>
<tr>
<td></td>
<td>Rpd3</td>
<td>HDAC 2</td>
<td>488</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>Nuclear</td>
</tr>
<tr>
<td></td>
<td>Rpd3</td>
<td>HDAC 3</td>
<td>428</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>nuclear/cyttopl.</td>
</tr>
<tr>
<td></td>
<td>Rpd3</td>
<td>HDAC 8</td>
<td>377</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>Nuclear</td>
</tr>
<tr>
<td>2 a</td>
<td>HDA1</td>
<td>HDAC 4</td>
<td>1,054</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>nuclear/cyttopl.</td>
</tr>
<tr>
<td></td>
<td>HDA1</td>
<td>HDAC 5</td>
<td>1,122</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>nuclear/cyttopl.</td>
</tr>
<tr>
<td></td>
<td>HDA1</td>
<td>HDAC 7</td>
<td>855</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>nuclear/cyttopl.</td>
</tr>
<tr>
<td></td>
<td>HDA1</td>
<td>HDAC 9</td>
<td>1,011</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>Nuclear</td>
</tr>
<tr>
<td>2 b</td>
<td>HDA1</td>
<td>HDAC 6</td>
<td>1,215</td>
<td>2</td>
<td>Zn$^{2+}$-dependent</td>
<td>nuclear/cyttopl.</td>
</tr>
<tr>
<td></td>
<td>HDA1</td>
<td>HDAC 10</td>
<td>669</td>
<td>2</td>
<td>Zn$^{2+}$-dependent</td>
<td>nuclear/cyttopl.</td>
</tr>
<tr>
<td>3</td>
<td>SIR2</td>
<td>SIRT 1-7</td>
<td>310–747</td>
<td>1</td>
<td>NAD$^{+}$-dependent</td>
<td>nuclear/cyttopl.</td>
</tr>
<tr>
<td>4</td>
<td>Rpd3/HDA1</td>
<td>HDAC 11</td>
<td>347</td>
<td>1</td>
<td>Zn$^{2+}$-dependent</td>
<td>Nuclear</td>
</tr>
</tbody>
</table>

(Source: Hildmann et al., 2007)
The accumulated literature on histone acetylation supports a general model in which histone acetylation contributes to the formation of transcriptionally competent environment by opening chromatin and allowing general transcription factors to gain access to the DNA template. Conversely, the classical role of HDACs is to reduce transcription by deacetylation of histone proteins, a process that makes the DNA bind to the histones more tightly (Fukuda et al., 2006). In addition, several non-histone proteins are regulated in their stability or biological function by the acetylation state of specific lysine residues (Gallinari et al., 2007). HDACs intervene in a multitude of biological processes and are part of a multiprotein family in which each member has its specialized functions. Eukaryotic HDACs are divided into four classes based on homology to yeast HDACs and phylogenetic analysis. Classes 1, 2, and 4 are closely related zinc-dependent enzymes, whereas class 3 HDACs or sirtuins are structurally unrelated nicotinamide adenine dinucleotide (NAD)-dependent deacetylase enzymes.

Class 1 enzymes are ubiquitously expressed and primarily located in the nucleus. Class 2 enzymes display a more tissue-specific expression in mammals and these enzymes shuttle in and out of the nucleus in response to cellular signals (Table 1.1). Many enzymes of these two classes act as transcriptional co-repressors by deacetylating nucleosomal histones (Hildmann et al., 2007). The activity of Class 4 HDAC is not well known.

1.13 The mammalian sirtuin enzyme family

a) Overview of the mammalian sirtuin family: classification, localization and function

Class 3 or the sirtuin family of histone deacetylases was named after their homology to the *Saccharomyces cerevisiae* gene silent information regulator 2 (Sir2) and includes 7 (SIRTs 1-7) enzymes. In mammals, there are seven members of the sirtuin family, Sirt1–7 that differ in their cellular localization and function (Haigis and Sinclair, 2010; Michan and Sinclair, 2007). The seven mammalian sirtuins share a highly conserved catalytic core domain but have differences in their N- and C-terminal ends (Frye, 2000). Based on phylogenetic analysis, mammalian sirtuins can be divided into four classes. Sirt1-3 belongs to class I, Sirt4 to class II, Sirt5 to class III and Sirt6, Sirt7 to class IV (Frye, 2000).
Sirtuins are protein deacetylases/ADP ribosyltransferases that target a wide range of cellular proteins in the nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation (SIRT1, -2, -3 and -5) or ADP ribosylation (SIRT4 and -6). The most important known function of sirtuins is their regulation of transcriptional repression, mediated through binding of a complex containing sirtuins and other proteins (Dali-Youcef et al., 2007). Numerous studies have shown that sirtuins have pathophysiological relevance to neurodegeneration, muscle differentiation, inflammation, obesity and cancer (Fulco et al., 2008; Albani et al., 2010; Zillikens et al., 2009; Kim et al., 2009; Yoshizaki et al., 2010).

b) Enzymatic activity of sirtuins

Although the initial activity of sirtuins was reported as NAD+-dependent ADP-ribosylation (Tanny et al., 1999), protein deacetylation is the most prevalent reaction that sirtuin enzymes catalyze. The deacetylation reaction occurs in two continuous stages to generate deacetylated protein, NAM and 2′-O-acetyl-ADP-ribose (2′-OAADPr) (Sauve et al., 2006; Tanner et al., 2000). In the first stage, sirtuins cleave NAD+ to produce NAM and the nucleophilic addition of the acetyl oxygen to C1′ of the ADP-ribose moiety to form C1′-Oalkylamidate intermediate (Sauve, 2010). The nucleophilic attack mechanism has been subject to debate between SN1 and SN2 type for the cleavage of the glycosidic bond between NAM and the rest of NAD+. NAM can inhibit sirtuins by rebinding to reverse the reaction through the base-exchange mechanism (Sauve et al., 2006).

In the second stage, the C1′-O-alkylamidate intermediate converts to the bicyclic intermediate by using the conserved Histidine as a general base to induce nucleophilic attack of the 2′-OH group of the ribose onto the iminium carbon of the O-alkylamidate intermediate. The crystal structure of the bicyclic intermediate between thiosuccinyl H3 peptide and NAD+ on Sirt5 was recently solved to provide an evidence for the mechanism (Zhou et al., 2012). The bicyclic intermediate is disrupted by a base activated water molecule to form deacetylated protein and 2′-O-acetyl-ADP-ribose (Sauve and Youn, 2012). Both 2′-O-acetyl-ADP-ribose and 3′-O-acetyl-ADP-ribose exist in equilibrium as solution products of sirtuins (Jackson and Denu, 2002).
c) Pharmacological inhibitors of sirtuins

While sirtuin activators have mainly been developed for Sirt1, sirtuin inhibitors have been studied on different sirtuin members including ySir2 (yeast Sir2), Sir2Tm (Thermotoga maritima sirtuin), and mammalian Sirt1, Sirt2, Sirt3 and Sirt5. The first sirtuin inhibitors are mostly based on substrates and products of the deacetylation reaction. NAM inhibits Sir2 activity by rebinding to attack the O-alkylamidate intermediate (Sauve and Schramm, 2003). Carba-NAD+ is a weak inhibitor of sirtuins (Landry et al., 2000). Thioacetyllysine derived peptides have been described as sirtuin inhibitors by hindering the reaction via formation of a stable S-alkylamidate intermediate instead of the native, transient O-alkylamidate intermediate (Smith and Denu, 2007). Moreover, the replacement of N-acetyl group with other groups and chemical modifications of peptide substrate have also reported as an approach to develop sirtuin inhibitors (Chen, 2011).

d) Pharmacological activators of sirtuins

The sirtuin activating compounds were called STACs. Subsequently, it is the polyphenol family and found resveratrol (3,4’,5-trihydroxystilbene) as the most potent activator candidate with ~13-fold increase in substrate deacetylation of Sirt1 and lower Km of the enzyme for the substrate and NAD+ (Howitz et al., 2003). Resveratrol, a naturally occurring polyphenol found in grapes and red wine, has been proposed to be responsible for the cardioprotective effects, invoked by the so-called ‘French Paradox’ (Renaud and de Largeril, 1992). Resveratrol, an antioxidant, is known as a multiple targets such as COXs (Pacholec et al., 2010). SIRT1 is found to be activated by resveratrol (Howitz et al., 2003), and the oral administration of resveratrol reduces plasma glucose and the triacylglycerol concentration in streptozotocin induced diabetic rats by 25% and 50% compared with vehicle-treated rats respectively (Su et al., 2006). Resveratrol promotes the deacetylation of PGC-1α by SIRT1, thereby reducing body weight and insulin resistance and increasing the aerobic capacity, motor function and survival of mice with high-fat-diet-induced obesity (Baur et al., 2006). Resveratrol, a natural polyphenol found in red wine and other plant-based foods, is able to mimic CR in anti-aging and possess many other benefits such as antivirus, anti-inflammation, anti-diabete and cardioprotective effects (Mohar, 2012).
Resveratrol was reported to extend lifespan of different organisms including yeast, worm and fly dependent on sirtuin activity (Howitz et al., 2003; Wood et al., 2004). This compound can also induce lifespan extension in fish but the relation of its effect to sirtuins is unclear (Valenzano et al., 2006).

In addition, many studies have emphasized that antioxidants have potential health promoting and disease preventing effects. N-acetyl cysteine (NAC) is an antioxidant that exhibits gene expression modifications helping to restore cells ability to fight damage from reactive oxygen species (ROS). N-acetyl cysteine (NAC), a potent antioxidant derives from the amino acid L-Cysteine and used clinically for decades for the treatment of many diseases. It has been shown that treatment with NAC by supplementing drinking water significantly increases the average and maximal lifespan, and reduces the rate of age-dependent associated disorders in mice (Kondratov et al., 2009). The therapeutic potential of NAC has been examined and is currently being further investigated across a range of illnesses as an antidote for specific toxins, as a bioprotective agent against oxidative stress and ischaemic injury and as a treatment for certain mental and physical illnesses (Seetal Dodd et al., 2008).

The discovery role of the sirtuins in oxidative stress and lifespan has led to a new and fascinating area of research in the field of antioxidants as several antioxidants including resveratrol and N-acetyl L-cysteine can activate sirtuins (Yu et al., 2009).

1. 14 Sirtuins – an important class of cellular regulators with a variety of functions

The sirtuins are a druggable class of enzymes (i.e. amenable to intervention by small molecules) that could have beneficial effects on a variety of human diseases (Chen et al., 2011).

a) SIRT1

SIRT1 seems to have a significant role in mammalian metabolism. It has been shown to deacetylate and thereby activate a crucial cofactor in mitochondrial biogenesis: PGC-1a (Rodgers et al., 2005). Mitochondrial activity in metabolically active tissues, such as muscle, will increase metabolic rate, drive glucose metabolism,
and thereby improve insulin sensitivity. Thus SIRT1 activation is a promising strategy for treating type 2 diabetes, obesity, and metabolic syndrome (Araki et al., 2004). It has been shown that SIRT1 regulates spermatogenesis at postnatal stages by controlling hypothalamus–pituitary gonadotropin signaling (Ullas Kolthur-Seetharam et al., 2009).

**b) SIRT2**

Tubulin deacetylation is the most frequently reported function of mammalian SIRT2 (North et al., 2003). A Crm-dependent nuclear export signal maintains the majority of the protein in the cytoplasm where it interacts with HDAC6 to regulate tubulin cytoskeletal structure. SIRT2 also plays a prominent role in mitosis, where it regulates mitotic exit from the cell cycle (Dryden et al., 2003), via association with the centrosome, mitotic spindle and midbody at different stages of the process (North and Verdin, 2007).

SIRT2 is dramatically downregulated in human gliomas (Allison and Milner, 2007), and ectopic overexpression of SIRT2 disrupts the tubulin network in cultured glioma cells, which reduces the number of stable clones, suggesting a function as a tumor suppressor gene (Hiratsuka et al., 2003). Conversely, others have reported p53 deacetylation and inactivation by SIRT2 after interaction with 14-3-3 (Jin et al., 2008). Given its ability to modulate the cell cycle, SIRT2 is increasingly being examined as a target for anti-cancer drugs (Neugebauer et al., 2008). It has also been demonstrated that SIRT2 is essential for normal embryogenesis and normal reproduction in both sexes (Imai and Guarente, 2010).

**c) SIRT6 AND SIRT7**

SIRT6 and 7 might also have metabolic functions. SIRT6 has been linked to base excision repair and glucose homeostasis (Mostoslavsky et al., 2006). SIRT6 knockout mice show increased DNA damage, have low levels of IGF1, and have a catastrophic decline in blood glucose beginning two weeks after birth. SIRT7 is the only mammalian sirtuin localized to nucleoli and has been shown to be a positive regulator of ribosomal RNA (rRNA) synthesis and ribosome biogenesis (Ford et al., 2006).
1. 14. 1 Mitochondrial sirtuins

Mitochondria are crucial intracellular organelles involved in energy production, metabolism, and intracellular signaling (Alberts, 2008). Mitochondrial number and/or activity change in response to a variety of physiological conditions such as nutrients, exercise, and change in temperature or oxygen levels, as well as during aging. Because they represent the main provider of cellular energy, altered mitochondria function can have a great impact upon the health of an organism (Schiff et al., 2011). In addition, the number of functional mitochondria is known to decrease with aging, so an increase in mitochondrial biogenesis could exert an anti-aging effect by buffering this decline (Cantó and Auwerx, 2009). Indeed, mitochondrial dysfunction has been linked to diseases including obesity, type 2 diabetes, and cancer, reproductive disease (Wallace, 2005; Wang et al., 2010). However, how mitochondrial dysfunction contributes to disease pathogenesis is not fully understood. In addition, mitochondria are a key control point for the regulation of steroid hormone biosynthesis. Any alteration in the mitochondria function leads to decreased steroidogensis (Christenson et al., 2001).

Mitochondria have been linked with infections and also diseases of reproductive and sirtuins might provide a key link between mitochondrial dysfunction, inflammation and metabolic disease. Thus, any regulator of mitochondrial function might be particularly relevant to metabolic disease. Mitochondrial sirtuins modulate physiological signals, in addition to their metabolic functions, and sirtuin activation provides benefits in many disease models. In the near future, sirtuin modulation will become a new tool for helping to overcome various diseases, although it might not be useful for generally extending the lifespan. It has been reported that SIRT3, SIRT4 and SIRT5 are found principally in the mitochondria plays a pivotal role in regulation of energy metabolism and endocrine pathways (De Moura et al., 2014).

a) SIRT3: The bonafide mitochondrial deacetylase

At least 20% of mitochondrial proteins are acetylated in proteomic surveys, and the importance of acetylation is suggested by the high conservation of many sites, from Drosophila to humans (Choudhary et al., 2009). In both prokaryotes and eukaryotes, reversible acetylation of metabolic enzymes appears to be crucial for the
regulation of metabolic flux in response to different sources of metabolic fuel or diverse metabolic states (Kim et al., 2006; Weinert et al., 2011).

In mice lacking SIRT3 shown increase global levels of protein acetylation in a variety of tissues (Lombard et al., 2007). Hyperacetylation at specific lysine sites of metabolic enzymes such as long chain acyl-CoA dehydrogenase (ACADL) and isocitrate dehydrogenase 2 (IDH2) have been linked to various metabolic phenotypes in SIRT3 null mice (Hirschey et al., 2011; Someya et al., 2010; Shulga and Pastorino, 2010). In a few cases, such as cyclophilin D, SIRT3 deacetylation instead impedes its function (Shulga and Pastorino 2010). It has been reported that Mn superoxide dismutase (SOD2) are deacetylated by SIRT3 under specific conditions: K53 and K89 during calorie restriction, K122 in response to ionizing radiation stress, and K68 in response to increased ROS levels (Chen et al., 2011). In addition, recent study demonstrated that SIRT3 knockout mice, leading to deficient SOD2 activation, and suggesting that SIRT3 activity is crucial for the deacetylation of these two key sites and the activation of SOD2 (Qiu et al., 2010; Kendrick et al., 2011; Schwer et al., 2009).

b) SIRT4: still a mysterious enzyme

SIRT4 is abundantly expressed in pancreatic β cells and is involved in the regulation of insulin secretion (Haigis and Guarente, 2006; Ahuja et al., 2007). However, its precise enzymatic functions remain unclear. It displays no detectable NAD-dependent deacetylase activity (Lombard et al., 2007) and may possess weak ADP-ribosyltransferase activity (Haigis and Guarente, 2006). However, this activity is more than 1000-fold slower than that of a bacterial ADP-ribosyltransferase, casting doubt on its physiological significance (Du et al., 2011). SIRT4 suppresses GLUD1 activity via ADP-ribosylation, in contrast to SIRT3, which activates GLUD1 via deacetylation. SIRT4-mediated inhibition of GLUD1 reduces the generation of ATP from the catabolism of glutamate and glutamine, which is essential for the ability of β cells to secrete insulin in response to amino acids. Mice lacking SIRT4 show correspondingly increased amino acid-stimulated insulin secretion (Nasrin et al., 2010).

In addition, SIRT4 interacts with insulin degrading enzyme (IDE) and the ADP/ATP carrier proteins ANT2 and ANT3 (Ahuja et al., 2007), although the implications of
these interactions remain unclear. Little is known of the functions of SIRT4 outside pancreatic β-cells, but there are more hints of an interesting contrast with SIRT3. In contrast to SIRT3, SIRT4 expression is reduced during CR and increased in mouse models of diabetes. SIRT4 is a negative regulator of FA oxidation in liver and muscle. Knockdown of SIRT4 expression both in vitro and in vivo increases the expression of genes involved in FA oxidation and oxidative phosphorylation, including SIRT1, medium chain acyl-CoA dehydrogenase (MCAD), carnitine palmitoyltransferase 1 (CPT1), PGC-1α, cytochrome c, ATP synthase, and IDH2, thereby enhancing FA oxidation and mitochondrial respiration. Interestingly, increased expression of SIRT1 in response to SIRT4 knockdown is required for the observed increase in FA oxidation (Nasrin et al., 2010). Nevertheless, how SIRT4 enzymatic activity in the mitochondrion affects gene transcription in the nucleus is unknown. Identifying the true enzymatic activity of SIRT4 and its mitochondrial target will undoubtedly shed light on its function.

c) SIRT5: the new frontier of protein deacylation

SIRT5 was an enzymatic enigma until the recent finding that it possesses unique, potent demalonylase and desuccinylase activities (Du et al., 2011; Peng et al., 2011). Malonyl-lysine and succinyl-lysine modifications have been identified in a variety of organisms ranging from yeast, worms, flies, and mice to humans (Zhang et al., 2011). The biological significance of lysine malonylation and succinylation is currently unknown. Many of the malonylated or succinylated proteins identified so far are important metabolic enzymes, including IDH2, serine hydroxymethyltransferase, glyceraldehyde 3-phosphate dehydrogenase, GLUD1, malate dehydrogenase 2, citrate synthase, carbamoyl phosphate synthetase 1 (CPS1), HMGCS2, thiosulfate sulfurtransferase, and aspartate aminotransferase (Du et al., 2011; Peng et al., 2011). How lysine malonylation and succinylation modulate the function of these enzymes has yet to be investigated. However, studying protein acetylation, and given the larger size and negative charge associated with malonylation or succinylation, one can reasonably expect that these modifications and their regulation by SIRT5 will play a significant role in metabolic regulation. In addition to these novel enzymatic activities, SIRT5 may also function as a protein deacetylase on the urea cycle enzyme CPS1, and
thereby increase its activity (Nakagawa et al., 2009). SIRT5 has also been shown to regulate CPS1 activity through desuccinylation (Du et al., 2011).

Of the seven mammalian sirtuins, SIRT3, 4 and 5 are located in mitochondria where they are not functionally redundant; instead, as described above, they exhibit distinct activities and their expression levels respond differently to changing metabolic states. As an orchestra of three, mitochondrial sirtuins may coordinate mitochondrial function through multiple regulatory layers of post-translational protein modifications in response to dynamic changes of nutrient availability and metabolic states.

1.15 Sirtuins and Reproductive function

Numerous studies have shown that sirtuins have pathophysiological relevance to neurodegeneration, muscle differentiation, inflammation, obesity and cancer (Fulco et al., 2008; Albani et al., 2010; Zillikens et al., 2009; Kim et al., 2009; Yoshizaki et al., 2010). Whereas very little is known about the role of sirtuins in regulating signaling pathways involved in reproductive tissues. It has been shown that SIRT1 regulates spermatogenesis at postnatal stages by controlling hypothalamus- pituitary gonadotropin signaling (Kolthur-Seetharam et al., 2009). It has also been demonstrated that SIRT2 is essential for normal embryogenesis and normal reproduction in both sexes (McBurney et al., 2003). However, there are no reports that demonstrate a role for sirtuins (an important member in regulation of many endocrine pathways) in regulating endocrine signaling of reproductive tissues. Hence, this present study aims to investigate the possible role of sirtuins, a member of class 3 HDACs in regulation of Leydig cell function.
Scope of the present study....
Scope of the present study....

Male urogenital tract is susceptible to gram-negative bacterial infections that produce a state of inflammation, particularly in the testis resulting in infertility or subfertility. Almost 15% of male infertility cases seen in fertility clinics are due to male accessory gland infections (Hales et al., 1999). The production of testosterone in testicular Leydig cells is essential for the maintenance of spermatogenesis and male fertility. Furthermore, the conversion of testosterone to estradiol is catalyzed by aromatase in steroidogenic tissues and approximately 60% of estradiol in circulation in men is derived from testicular secretion (de Ronde and de Jong, 2011). The increased production of cytokines in inflammatory disease leads to inhibition of Leydig cell steroidogenesis and results in male infertility (Sadasivam et al., 2015).

Bacterial lipopolysaccharide (LPS) is the most important contributing factor in pathogenesis of bacterial infection in male reproductive tissues. Infection and inflammation can be reproduced in both in vitro and in vivo by administration of LPS, and several studies have reported that administration of LPS or with septic agents that generate LPS in animals causes inhibition of testicular steroidogenesis (O’Bryan et al., 2000; Allen et al., 2004; Sharma et al., 1996). LPS mediated production of pro-inflammatory cytokines has an inhibitory role in steroidogenesis through the production of increased reactive oxygen species (ROS) (Reddy et al., 2006). LPS generated ROS disrupts mitochondrial membrane permeability and thus inhibits Leydig cell function (Allen et al., 2004). It has also been demonstrated that LPS treatment is associated with apoptosis of testicular Leydig cells and germ cells (Metukuri et al., 2010; Wu et al., 2012). Several studies have reported that LPS plays a key role in the regulation of steroidogenic enzymes gene expression including 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Magata et al., 2014; Sadasivam et al., 2014). However, the exact mechanisms by which LPS causes impaired steroidogenesis and apoptosis are not well studied.

Sirtuins (SIRT1-7) are group of deacetylases/ADP ribosyltransferases that target a broad range of cellular proteins for post-translational modifications by acetylation or ADP ribosylation to regulate gene expression in various tissues.
Numerous studies have shown that sirtuins play a pivotal role in pathophysiology of cancer, obesity, neurodegeneration, inflammation, muscle differentiation and aging (Dali-Youcef et al., 2007; Shoba et al., 2009). It has been shown that SIRT3, SIRT4 and SIRT5 are found predominantly in the mitochondria and have role in regulation mitochondrial functions. Of these mitochondrial sirtuins, SIRT4 functions as an effective ADP-ribosyltransferase and has role in fatty acid metabolism (Nasrin et al., 2010), insulin secretion (Ahuja et al., 2007) and tumor suppression (Jeong et al., 2013). In addition, SIRT4 has been reported to regulate ATP homeostasis by mitochondrial uncoupling of the adenine nucleotide translocator 2 (ANT2) (Ho et al., 2013). Anti-apoptotic activity of SIRT4 has been demonstrated to protect cardiomyoblast cells from hypoxia (Liu et al., 2013). Most recently, it has been shown that LPS mediated suppression of SIRT4 aggravates the induction of pro-inflammatory cytokines in human umbilical vein endothelial cells (Tao et al., 2014).

Despite these emerging findings regarding the physiological role of sirtuins, there are no reports that demonstrate the role for sirtuins in testicular function. Since, the early reactions of testicular steroidogenesis occur in mitochondria, we speculate that sirtuins may have a role in the regulation of steroidogenesis.

The present study is aimed to investigate the effect of bacterial LPS induced inflammation on sirtuins and their implications in testicular Leydig cell function. The study involves the following specific objectives.

- To determine the effect of inflammation on Leydig cell steroidogenesis and cellular apoptosis.
- To investigate the role of sirtuins in regulation of lipopolysaccharide mediated Leydig cell dysfunction.
- To find the effect of neutaceuticals on sirtuins and their role in regulation of lipopolysaccharide mediated Leydig cell dysfunction.